

Optimization Extraction of *Xylocarpus granatum* Stem as Antioxidant and Antiglycation

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ABSTRACT

Xylocarpus granatum is an Indonesian plant that has bioactive compounds such as phenolic and has high antioxidant activity. The aim of this research was to determine the optimum maceration condition for *X. granatum* stem as antioxidant and antiglycation. The optimum conditions of maceration were affected by the extraction variables (concentration, sample/solvent ratio, extraction time), and could be evaluated by using surface response method. The optimum condition of maceration is predicted to be achieved when the ethanol concentration as solvent is 52.25%, the extraction time is 15.92 hours, sample/solvent ratio is 1 g/9 ml with the responds of yield, total phenol content, flavonoid, inhibition for 2,2'-diphenylpicryl hydrazyl, inhibition capacity of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and inhibition antiglycation were 12.81%, 1.95 mg of gallic acid/g extract, 62.33 µg quercetin/g extract, 41.11%, 0.71 TEAC, and 112.33%, respectively. Optimization extraction conditions shows that the extraction variables have significant effect on responds so it can reduce the extraction time, produce high bioactive constituents, and reduce the production cost.

1. Introduction

Indonesia is a tropical country that has a lot of UV exposure from sunlight. This sunlight can accelerate free radicals' production. Free radicals can accelerate skin aging and increase the activity of the tyrosinase enzyme which is responsible for melanogenesis or hyperpigmentation (Batubara and Adfa 2013). Halliwell (2001) in Yagi *et al.* (2013) reported that free radicals play a role in the formation of advanced glycation end products (AGEs). Glycation is a nonenzymatic reaction between reducing sugars and amino groups of proteins, nucleic acids, or phospholipids to form AGEs (Yeh *et al.* 2017). The effects of AGEs, recent studies have also addressed on many chronic diseases and aging-related disease in clinical practice (Rabbani *et al.* 2016). Antiglycation has been considered as an effective strategy to slow down human aging and disease development (Yeh *et al.* 2017).

Antioxidants can be used to prevent skin aging (Thornfeldt and Bourne 2010). One of antioxidant substances is polyphenol. Polyphenols can inhibit the

biosynthesis of AGEs through antioxidant properties, metal-chelating ability, protein interaction, and blocking the receptor for advanced glycation end products (AGEs) (Chen *et al.* 2006). Plants can be used as a source of natural antioxidants (Bernatoniene *et al.* 2011). *Xylocarpus granatum* is a mangrove plant which has phenolic bioactive compounds, alkaloids, steroids (Kopkol *et al.* 1990; Bandaranayake 2002), and triterpenoids (Gazali *et al.* 2014). According to Batubara *et al.* (2010), the stem part of *X. granatum* can be a source of antioxidants and whitening agent. In addition to the stem, seed and fruit peel of *X. granatum* can be used as a source of antioxidants (Zamani *et al.* 2015), but stem part has the highest antioxidant activity and tyrosinase inhibitors (Darusman *et al.* 2011; Gazali *et al.* 2014; Zamani *et al.* 2015).

Extraction optimization was carried out to obtain the optimum extraction conditions. Extraction can be influence by several factor such as extraction time, variation in solvent concentration, and sample ratio with solvent. Determination of the optimum extraction conditions is carried out using the surface response method. Responses were reviewed based on the acquisition of total phenol content, total

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flavonoids, antioxidant activity, and antiglycation activity of *X. granatum* stem. The aim of this research was to determine the optimum maceration condition for *X. granatum* stem as antioxidant and antiglycation.

2. Materials and Methods

2.1. Plant Materials

X. granatum stem from Togean, Central Sulawesi.

2.2. Chemicals and Instruments

The chemical used are 2,2'-diphenylpicryl hydrazyl (DPPH), ethanol, methanol, bovine serum albumin (BSA), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), glucose, fructose, Folin-Ciocalteu's reagent, gallic acid, ascorbate acid, and quercetin. The instrument used are ELISA plate well reader (Merk Biotek Epoc Spekro UV-Vis), and fluorometer (FluoroStar BMG Labtech).

2.3. Design of Experiments for *X. granatum*'s Extraction

The Central Composite Design (CCD) method (Table 1) was used in order to optimize seven different response variable; yields, phenolic content, flavonoid content, antioxidant (DPPH and ABTS), and antiglycation.

2.4. Total Phenolic Content (TPC)

Total phenolic content was determined according to the previous method (Premakumara *et al.* 2013). Briefly, 20 µl extract or standard solution (gallic acid)

of different concentrations (0-100 mg/l) was added 110 µl Folin-Ciocalteu's reagent to the well plate. Sodium carbonate (70 µl) was added to the mixture. After incubation at room temperature for 30 minutes, the absorbance was measured at 765 nm using a ELISA reader. Total phenolic content of the samples were determined by plotting the absorbance data of sample into calibration curve of gallic acid. The results were then expressed as gallic acid equivalents (GAE) (% (w/w)) of extract.

2.5. Total Flavonoid Content (TFC)

Total flavonoid was determined as described by Lee *et al.* (2011). Extract or standard solution (10 µl) was added into the well plate containing 60 µl methanol, 10 µl AlCl₃ 10%, 10 µl CH₃COONa 1 M, and 120 µl water. The mixture was incubated at room temperature for 30 minutes, the absorbance was measured at 415 nm. Quercetin was used as a standard solution. The results were expressed as quercetin equivalent (% (w/w)) of the extract.

2.6. Antioxidant Activities

DPPH radical scavenging activity. The experiment was conducted as described by Batubara *et al.* (2015) with some modifications. DPPH solution (100 µl) (125 µM) was reacted with 100 µl sample (500, 250, 125, 62.5, and 31.25 µg/ml). The mixture was then incubated for 30 minutes. After incubation, the absorbance was read at 517 nm. Ascorbic acid was used as positive control, while ethanol was used as solvent and negative control.

ABTS. The experiment was conducted as described by Ree *et al.* 1999 with some modifications. ABTS solution 10 ml (7 mM) was oxidized by 5 ml potassium peroxide sulfate (2.55 mmol/l) for 16 hours. Further, about 180 µl ABTS radical was reacted with 20 µl sample and incubated for 15 minutes at microplate 96 well. The absorbance was measured at 734 nm. The results were expressed as Trolox Equivalents Antioxidant Capacity (TEAC) % w/w.

2.7. Antiglycation

Antiglycation was measured according to the method described by Povichit *et al.* (2010) with a slight modification. The reaction consisted of 80 µl BSA (20 mg/ml), 40 µl glucose (235 mM), 40 µl fructose (235 mM), and 80 µl of sample or positive control in 200 µl phosphate buffer 0.2 M (pH 7.4). The solution was incubated for 40 hours at 60°C, and then the excitation fluorescence intensity was measured at 370 nm and the emission at 440 nm. Aminoguanidine was applied as a positive control. Sample and positive control corrected

Table 1. Design of experiments central composite design

Run order	Ethanol concentration (%)	Extraction time (hours)	Ratio sample:solvent (g:ml)
1	0	24	1:3
2	0	16	1:6
3	40	16	1:3
4	40	8	1:6
5	80	8	1:3
6	40	16	1:6
7	40	16	1:6
8	40	16	1:6
9	80	8	1:9
10	40	16	1:6
11	40	16	1:6
12	40	16	1:6
13	0	8	1:9
14	80	16	1:6
15	40	24	1:6
16	40	16	1:9
17	0	24	1:9
18	0	8	1:3
19	80	24	1:3
20	80	24	1:9

solution were prepared same like sample but water used to replace the sample or positive control. Antiglycation activity was measured by using the following equation:

$$\% \text{ inhibition} = \left[1 - \frac{C-D}{A-B} \right] \times 100\%$$

where,

- A :fluorescence intensity of control solution
 B :fluorescence intensity of control corrected solution
 C :fluorescence intensity of sample solution
 D :fluorescence intensity of sample corrected solution

3. Results

The yield of extracts from the *X. granatum* stem of 20 run order ranges from 4.33% to 12.88% (Table 2). The ethanol 80% extract, extraction time 24 hour, and ratio sample:solvent (1:9) gave the highest yield. This indicates that stem contained semi-polar compound rather than more polar compounds. The content of the extracts will directly correlate with the activity. Different constituents will give different activities. Total phenolic of 20 run order extracts and its extract were expressed as miligram gallic acid equivalent/gram extract. The amount of total phenolic contents ranged from 0.57 to 1.89 mg gallic acid/g extract (Table 2). The ethanol 40%, extraction time 16 hour, and ratio sample:solvent

Table 2. Extraction yield, total phenolic, and total flavonoid contents of *X. granatum* stem extracts

Run order	Extraction yield (%)	Total phenolic (mg gallic acid/g extract)	Total flavonoid (µg quercetin/g extract)
1	4.33±1.26 ^j	0.30±0.01 ^k	23.36±0.74 ^g
2	5.49±1.04 ^{hij}	0.20±0.02 ^l	25.86±3.09 ^g
3	6.76±1.81 ^{fg hij}	1.10±0.02 ^g	27.11±10.98 ^{fg}
4	6.90±1.03 ^{fg hij}	1.43±0.03 ^d	23.66±1.51 ^g
5	4.84±3.31 ^{ij}	0.84±0.03 ^h	25.15±2.58 ^g
6	10.33±2.24 ^{abcd}	1.37±0.02 ^{de}	60.26±1.91 ^b
7	9.01±1.78 ^{cdef}	1.32±0.03 ^{ef}	46.03±0.72 ^d
8	7.02±1.65 ^{fg hi}	1.02±0.02 ^g	37.75±4.06 ^e
9	9.63±0.84 ^{bcdef}	1.79±0.03 ^b	52.26±1.32 ^{cd}
10	10.77±3.08 ^{abc}	1.75±0.03 ^b	61.75±2.16 ^{ab}
11	11.28±0.73 ^{abc}	1.53±0.04 ^c	46.32±8.12 ^d
12	11.17±0.95 ^{abc}	1.43±0.04 ^d	51.04±1.88 ^{cd}
13	7.37±0.32 ^{efghi}	0.66±0.01 ^{ij}	32.99±2.61 ^{ef}
14	8.73±1.78 ^{cdefgh}	1.57±0.05 ^c	60.19±4.78 ^b
15	9.96±0.15 ^{bcde}	1.24±0.12 ^f	50.36±0.17 ^{cd}
16	12.47±0.71 ^{9ab}	1.89±0.06 ^a	56.62±3.69 ^{bc}
17	7.77±0.83 ^{defgh}	0.61±0.03 ^j	47.74±2.60 ^d
18	6.07±0.55 ^{ghij}	0.57±0.01 ^j	25.69±1.13 ^g
19	4.25±0.42 ^j	0.71±0.09 ⁱ	21.08±0.34 ^g
20	12.88±0.79 ^a	1.72±0.16 ^b	67.31±5.01 ^a

Data given as mean ± standard deviation of triplicate test. Data followed by the same letter are not significantly different according to Duncan's multiple comparison test. P=0.01

(1:9) gave the highest total phenolic contents. Total flavonoid of 20 run order extracts and its were expressed as microgram quercetin/gram extract. The amount of total flavonoid contents ranged from 21.08 to 67.31 µg quercetin/g extract (Table 2). The highest total flavonoid contents in 80% ethanol extract was extracted for 24 hours with ratio sample:solvent (1:9).

The radical scavenging activity was determined by DPPH radicals and ABTS radicals, which are commonly used for assessment of radical scavenging activity *in vitro* and results are given in Table 3. All run order exhibited significant and dose dependent radical scavenging activity against both radicals. Measurement of antioxidant activity using DPPH method is expressed as % inhibition values of *X. granatum* stem extract at a concentration of 100 ppm. The % inhibition DPPH ranged from 17.40 to 70.92% (Table 3). The concentration of solvent with 40% ethanol, extraction time 8 hours, and ratio sample:solvent (1:6) has the highest activity among all samples, although the activity is not as high as ascorbic acid as positive control (Table 3).

The ability of the antioxidant activity of the ABTS method is reported by TEAC (Trolox Equivalent Antioxidant Capacity). The ABTS reducing property is directly proportional to the amount of phenolics, therefore, the potent ABTS reduction observed in this assay might be due to the phenolics and flavonoids

Table 3. Antioxidant and antiglycation activity of *X. granatum* stem extracts

Run order	% Inhibition DPPH (%)	% Inhibition ABTS (TEAC)	% Inhibition antiglycation (%)
1	17.40±2.87 ^j	0.21± 0.010 ^j	78.66±22.79 ^{cde}
2	26.02±4.67 ^{ij}	0.27±0.021 ⁱ	73.62±7.70 ^{de}
3	57.25±15.57 ^b	0.37±0.001 ^g	102.48±29.03 ^{abcd}
4	70.92±3.38 ^a	0.38±0.001 ^g	84.94±3.41 ^{cde}
5	55.26±0.94 ^{bc}	0.27±0.005 ⁱ	84.11±6.48 ^{cde}
6	50.13±3.69 ^{bcde}	0.57±0.001 ^c	74.53±1.74 ^{de}
7	36.87±7.91 ^{ghi}	0.50±0.001 ^e	78.38±4.38 ^{cde}
8	51.28±4.68 ^{bcd}	0.39±0.001 ^g	99.35±6.04 ^{bcd}
9	54.92±9.18 ^{bc}	0.54±0.001 ^d	131.66±3.04 ^a
10	42.92±9.18 ^{defg}	0.59±0.003 ^{bc}	83.12±3.52 ^{cde}
11	28.72±6.01 ^{hi}	0.61±0.017 ^b	97.70±31.29 ^{bcd}
12	31.78±8.67 ^{ghi}	0.60±0.031 ^b	80.52±11.17 ^{cde}
13	41.94±1.85 ^{defg}	0.39±0.020 ^g	77.96±4.53 ^{de}
14	43.16±0.53 ^{defg}	0.48±0.002 ^e	120.72±17.44 ^{ab}
15	37.38±6.29 ^{ghi}	0.53±0.027 ^d	72.82±9.13 ^{de}
16	32.88±3.32 ^{ghi}	0.69±0.001 ^a	101.55±27.60 ^{abcd}
17	41.94±1.21 ^{defg}	0.42±0.002 ^f	55.11±9.07 ^e
18	37.18±2.33 ^{ghi}	0.32±0.004 ^g	56.34±11.37 ^e
19	39.17±8.69 ^{efgh}	0.23±0.001 ⁱ	99.28±17.52 ^{bcd}
20	45.46±1.93 ^{cdef}	0.70±0.001 ^a	110.59±33.56 ^{abc}
Ascorbic acid	83.65±0.40	-	-

Data given as mean ± standard deviation of triplicate test. Data followed by the same letter are not significantly different according to Duncan's multiple comparison test. P=0.01

compounds present in extract. The TEAC of ABTS is ranged from 0.21 to 0.70 TEAC (Table 3). The highest TEAC value is found in the extract that macerated with 80% ethanol for 24 hours with a ratio of sample weight:solvent volume (1:9).

Antiglycation activity of 20 run order *X. granatum* stem extracts is given in Table 3. It showed that the glycation inhibition of all extract is more than 50% (ranged from 55.11 to 120.72% at 100 ppm concentration of extract). The highest % inhibition of extracts was at run order no 14, which was extraction with 80% ethanol, ratio sample:solvent (1:6), and extraction time 16 hours.

In the light with variance analysis result, it can be said that the triple interaction of yield, concentration solvent, and extraction time is found to be the most significant to yield of extraction. The response surface of this interaction is given in Figure 1. Figure 1a shows that maximum level of yield found between 25 and 50% of ethanol concentration and between 15 and 20 hours of extraction time. Figure 1b and c shows that high level of the yield found between 25 and 50% ethanol and between 20 and 25 hours of extraction time. The

optimum conditions of three factors for all responds can be seen in Table 4. The optimum conditions for each response are different, so to accommodate all responses, a point combination is drawn to describes the ideal conditions of all responses. The optimum extraction condition to produce the highest response was predicted by the model at 52.25% concentration, extraction time for 15.92 hours, and sample/solvent ratio of 1 g/9 ml. The optimum condition is expected to produce all responds as shown in Table 4.

Validation of the optimum conditions is performed by evaluate the responds of extract that produced in optimum condition. The results showed that the response value is not significantly different with predicted as shown in Table 4.

4. Discussion

The phenolic content of 20 run order extracts gave the highest content at ethanol 40% extract. It means that phenolic is effectively extracted with combination of ethanol and water. With this concentration of ethanol

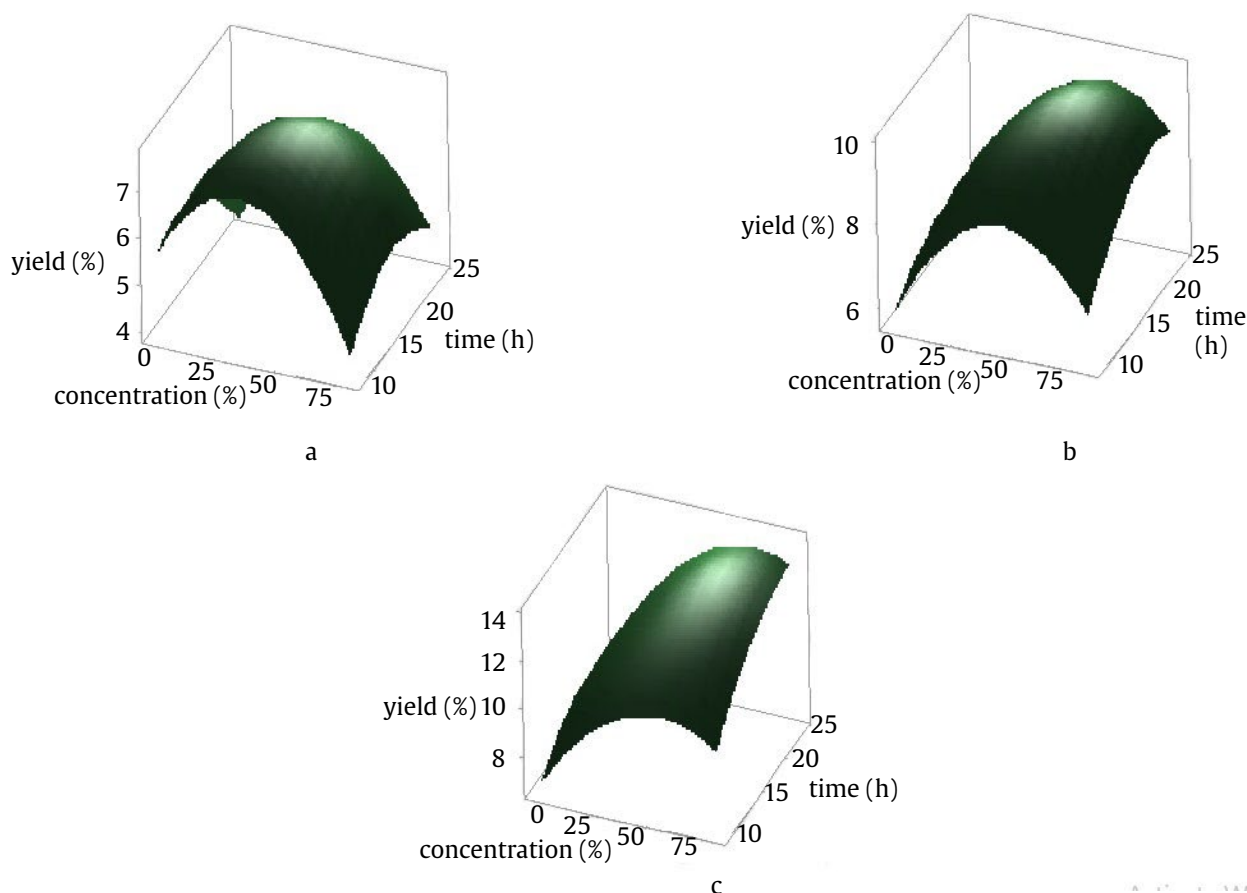


Figure 1. The responds surface of triple interaction of yield (%), concentration of ethanol (%), and extraction time (h) with ratio of sample to solvent of 1:3 (a), 1:6 (b), and 1:9 (c)

Table 4. Prediction and validation test results for *X. granatum* stem extracts

Respon	Predicted	Validation
Yields	12.81%	15.27%
Total phenolic	1.95 mg gallic acid/g extract	1.13 mg gallic acid/g extract
Total flavonoid	62.33 µg quercetin/g extract	56.73 µg quercetin/g extract
DPPH	44.11%	44.76%
ABTS	0.71 TEAC	0.54 TEAC
Antiglycation	112.33%	116.05%

as solvent for extraction, plant cell wall will easier to degrade and phenolic compounds will be easier to get out of plant cells (Tiwari *et al.* 2011). Flavonoid is one group of phenolic compounds that widely distributed in plant parts such as leaves, seeds, bark, and flowers and can act to scavenging the reactive oxygen species. Therefore, they generally possess as a good antioxidant and their effects on human nutrition and health are considerable (Orhan *et al.* 2007). According to Chang *et al.* (2009) *X. granatum* is a species contains flavonoid compound which are potent as antioxidant.

The antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (Moyo *et al.* 2010). It means that antioxidant activity from plants is closely related to the total content of phenolic and flavonoids (Naik *et al.* 2003). However, antioxidant capacity does not always positively correlate with total phenolic content (Scalzo *et al.* 2005). DPPH and ABTS both are radical agent but had a slight difference in the results. It because they have different types of free radicals and sensitivity (Shalaby and Shanab 2013; Zahra *et al.* 2016).

Phenolic compounds are also play an important role in the process of anti-glycation that lead to inhibit aging process, because its ability to reduce free radicals (Suwannalert *et al.* 2012; Sharafzadeh 2013). Antioxidants can inhibit the formation of AGEs. However, the anti-glycation activity does not always positively correlate with antioxidant activity, because both activities have different mechanism reactions. Our results revealed a strong positive correlation of antiglycation with total flavonoid contents and ABTS radical scavenging of *X. granatum* stem extract, especially at run order of 20. Findings of their study support the evidence that phenolic compounds significantly inhibit the protein glycation (Peng *et al.* 2008).

Validation results were found to be very close to the prediction. This modeling provides information that extraction can be carried out with a short time extraction, optimum solvent concentrations, and optimum ratio of sample and solvent. Variable solvent concentration, extraction time, and ratio sample:solvent had significant effect on yield, total phenolic contents, DPPH and ABTS methods antioxidant activity, and antiglycation activity. The optimum extraction conditions obtained were ethanol concentration as solvent of extraction 52.25%, ratio sample:solvent (1 g:9 ml), and extraction time for 15.92 hours. This optimum condition will reduce the processing time (extraction time), produce high yield of extraction with high bioactive constituent, and will reduce production cost especially extraction cost.

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